SIMULATED MOVING BED CHROMATOGRAPHY AND ITS ADAPTATION FOR PROTEIN PURIFICATION

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ABSTRACT

Approximately half of the cost associated with producing drug candidate proteins for early stage clinical trials can be attributed to the supply of chromatographic resin.[1] Continuous chromatography maximizes resin usage, thereby reducing these costs.[1] Two batch processes were well characterized and subsequently converted to continuous mode, the first being an enzyme polishing process using hydrophobic interaction chromatography, and the second the capture step of an enzyme using a customized affinity resin. Both processes were straightforward to design and yielded relatively high purity and acceptable yield. These results encourage the exploration of continuous chromatography for a wide variety of applications.

Readers: Dr. Marc Donohue, Dr. Honggang Cui

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INTRODUCTION

A large portion of the cost associated with producing drug candidate proteins for early stage clinical trials can be attributed to the supply of chromatographic resin.[1] It is often the case that before a resin has approached its cycle lifetime, the drug candidate is deemed unsuccessful and the customized resins rendered useless.[1] Reducing the amount of resin needed to produce purified drug candidates in early stage processes can therefore significantly decrease the material and economic waste associated with failed drug candidates.[1]

Chromatographic processes used in protein purification are typically run in batch mode. For flowthrough separations in this mode, product can begin exiting the column before the resin has reached its total binding capacity. Continuous chromatography makes use of multiple columns in series so that as breakthrough commences, protein can be salvaged on the following column, allowing the previous column to be loaded to maximum capacity.

The aim of the two studies discussed in this essay was to show that successful continuous chromatography methods are not extremely labor-intensive to design and can be worth exploring for many chromatographic separations.

BACKGROUND: TRADITIONAL CHROMATOGRAPHY

Liquid chromatography is a technique used to separate compounds in solution based on differences in their physical properties, such as size, charge, polarity, or even ligand-specific binding.[2] Based on how the components differ, a specific type of resin is selected and packed into a column. The solution is then pumped through the column, and due to the components' different affinities to the resin, they travel through the column at different rates and exit at different times, allowing for them to be collected separately.

TYPICAL CHROMATOGRAPHIC METHODS

Before the resin can be used for separation, it must first be equilibrated using a buffer safe for both it and the feed.[3] The feed can then be loaded onto the column and the column washed with more buffer to remove any unbound components.[3] Depending on how strongly the compounds interact with the resin, they will either exit over time with the same buffer, or they will require a separate desorbant. After elution, the resin can be stripped to remove any remaining components, sanitized to prevent contamination, and stored in a solution to preserve it for future use. This general method is summarized in Figure 1.

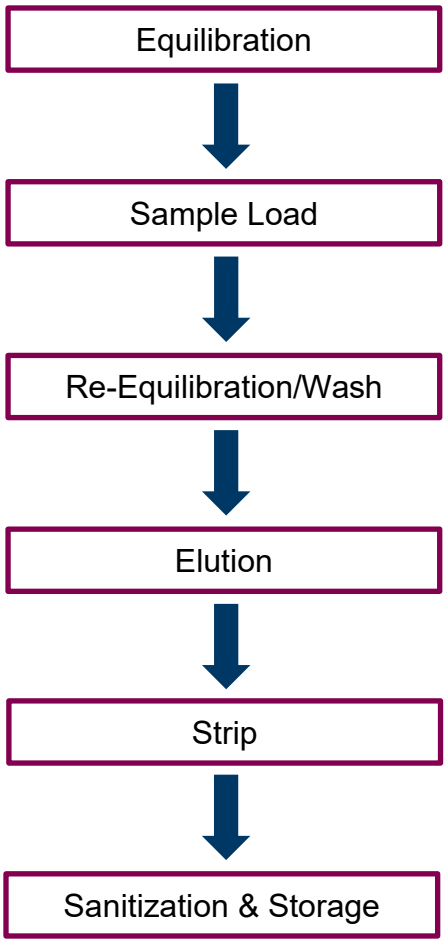

Figure 1. General Chromatographic Method
Chromatographic resins must be equilibrated before the feed can be loaded. After loading, the resin is washed to remove unbound material. If required, a separate desorbant is used to elute components of interest and the resin is stripped to clear all binding sites. If the resin is unused for long periods of time, it should be sanitized and stored in appropriate buffers.[3]

Throughout the method, the chromatography system can measure the UV absorbance and the conductivity of the fluid exiting the column. Spikes in UV

readings typically indicate the presence of protein, while those in conductivity indicate the presence of salt. The volumes of buffer required for the equilibration and re-equilibration steps are simply the amount needed for the UV and conductivity readings to stabilize over time, signifying that the entire column is saturated with buffer. Again, in the elution step, the presence of protein in the flowthrough is detected via peaks in UV 280 or 215 nm readings, and so a constant measurement after the elution peak(s) signals that the method can progress to the next step. It is usually simpler to first use a method with steps that are significantly longer than expected, and from the resulting chromatogram decide what volume is actually needed for the UV and conductivity readings to stabilize. From this information a successful batch method can be determined for the separation.

CHROMATOGRAPHY IN PROTEIN PURIFICATION

Proteins are often promising drug candidates and therefore must be purified before they can be tested in patients. It is common that several chromatography steps are used in sequence to achieve high purity. First, an affinity resin using ligand-binding interactions is specifically selected to bind the protein of interest with few impurities, allowing the many contaminants found in the protein's media to flow through. This is referred to as the protein's capture step.[1] Because

the impurities that remain tend to be structurally similar to the protein of interest, the following polishing steps must target the impurities directly using other modes of chromatography.[1] The number of polishing steps required to sufficiently purify the protein can vary depending on how many impurities remain and how difficult they are to remove.

BENEFITS OF CONTINUOUS CHROMATOGRAPHY

In batch flowthrough separations, components can begin eluting before the resin has reached maximum capacity, limiting the amount of feed that can be loaded at once. Continuous chromatography addresses this problem by connecting columns in series so that as the first column is loaded, the following column will retain the flowthrough. The first column can therefore be loaded to maximum capacity without losing product. In addition to improvements in resin usage, continuous chromatography converts the time-dependence of batch chromatography to space-dependence, allowing the process to run without stopping, and potentially without supervision.

CONTINUOUS CHROMATOGRAPHY IN THEORY: TRUE MOVING BED (TMB) CHROMATOGRAPHY

In order to understand how continuous chromatography systems are used in practice, one must first understand the theory and design behind them. The ideal, truly continuous model is known as true moving bed (TMB) chromatography. In this model, one can imagine a bed of resin of infinite length with an applied velocity towards the left. Feed can then be added from above, as represented in Figure 2.[3]

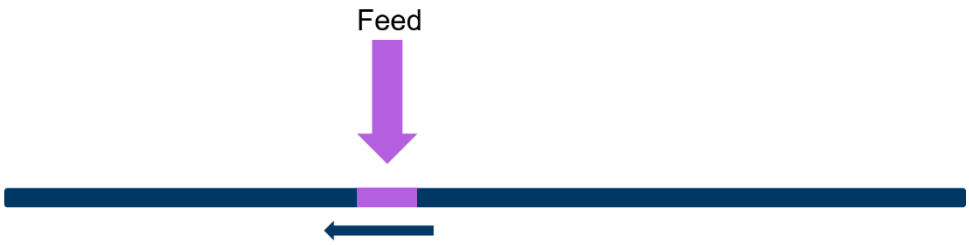

Figure 2. True Moving Bed Theory: Adding Feed to a Bed of Infinite Length
The theory of true moving bed chromatography begins with a bed of resin of infinite length (navy blue) moving to the left. A two-component feed (purple) is added from above.[3]

If buffer is then forced from the left through the bed, the velocity of the feed's components will be the sum of the velocity of the components relative to the bed and the bed's velocity relative to a stationary frame. Because the feed's

components interact differently with the resin, their velocities relative to the bed will differ; these values can be determined from batch studies. The velocity of the bed can then be selected so that the net velocity of the faster-moving component is positive and that of the slower-moving component is negative. Practically, this means that the desired and undesired components will move in opposite directions, as depicted in Figure 3.[3]

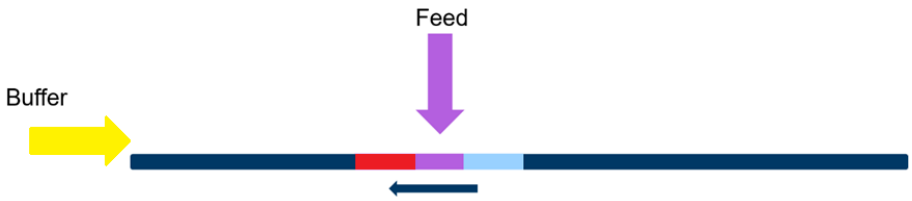

Figure 3. True Moving Bed Theory: Separating Components in Space
If buffer is pumped through the resin and the velocity of the bed of resin has an absolute value intermediate to the velocities of the two components relative to the bed, the slower-moving component (red) will move to the left and the faster-moving component (blue) will move to the right, and so they will be separated in space. [3]

The components are therefore separated in space. If outlets were added fixed relative to a stationary frame, they could be captured separately, with the faster-moving component collecting in the raffinate and the slower-moving component in the extract, as shown in Figure 4.[3]

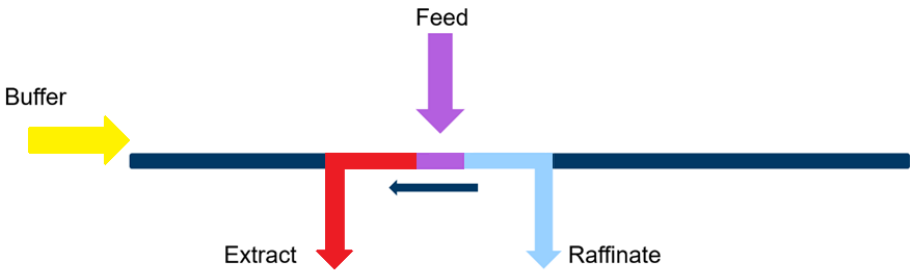

Figure 4. Complete True Moving Bed Theory: Component Collection
If outlet ports are added to the true moving bed system, the separated components can be collected continuously.[3]

It is important to note that there are several requirements in this model that are impossible to meet in practice, namely:

- There cannot be a bed of infinite length.
- It is not possible to have a buffer feed travel ahead of this bed indefinitely.
- In order to maintain fixed inlet and outlet ports in space, every portion of the resin would need to be accessible, preventing the buildup of pressure required and creating contamination concerns, etc.

Figure 5 displays how the translation of this bed to a circular, rotating configuration solves the first two of these problems.

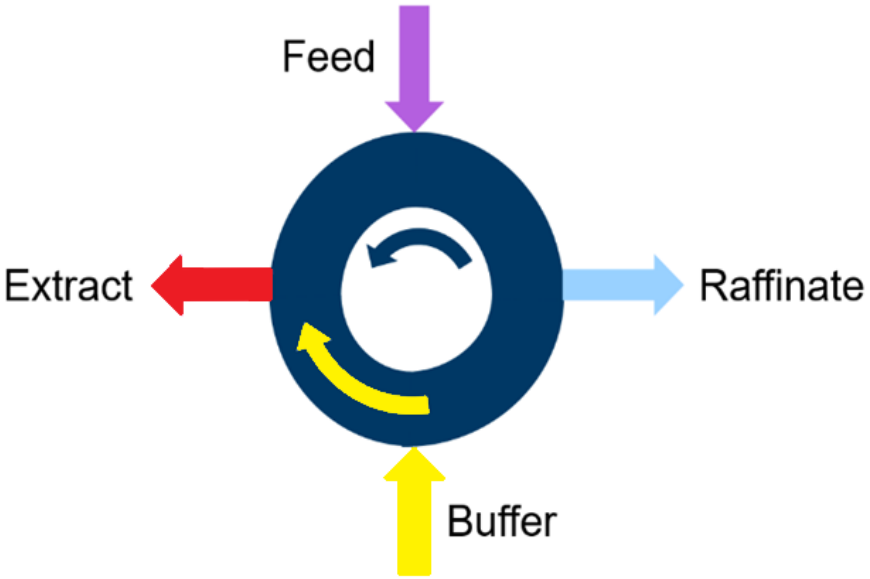

Figure 5. True Moving Bed in a Circular Configuration
Converting the original true moving bed design to a circular configuration eliminates the need for a bed of infinite length and for the buffer inlet to travel indefinitely.[4] The yellow arrows denote the movement of the buffer, while the navy blue arrow denotes to rotation of the resin.

Despite these improvements, the requirement of having every portion of the resin be externally accessible still remains, and so this setup cannot be used as is in practice. Simulated moving bed chromatography modifies true moving bed chromatography to address this concern.

CONTINUOUS CHROMATOGRAPHY IN PRACTICE: SIMULATED MOVING BED (SMB) CHROMATOGRAPHY

Simulated moving bed (SMB) chromatography addresses the final concern from TMB chromatography by using multiple short beds of resin in series rather than a single long bed. Inlets and outlets are stationed between them, and instead of the beds moving continuously, they can be imagined shifting positions countercurrent to the mobile phase after a designated amount of time, known as the switch time.[4]

MIMICKING A MOVING BED USING A VALVE BLOCK

The actual practice of SMB chromatography makes use of the fact that successful separation depends on the *relative* motion of the fluid flow and column movement: The result is the same if the columns are moved backward or if the inlets and outlets are moved forward. In order to avoid physically rotating and connecting/disconnecting the inlets and outlets with each switch, a valve block is instead used to redirect the flow of the buffer, feed, extract, and raffinate, mimicking the forward movement of the inlets and outlets, and producing the same products that would have resulted from moving the columns backward.[4]

ZONE ROLES IN SEPARATION

The buffer, feed, extract, and raffinate divide the columns into zones as shown in Figure 6. Each zone plays a specific role in separation.[4]

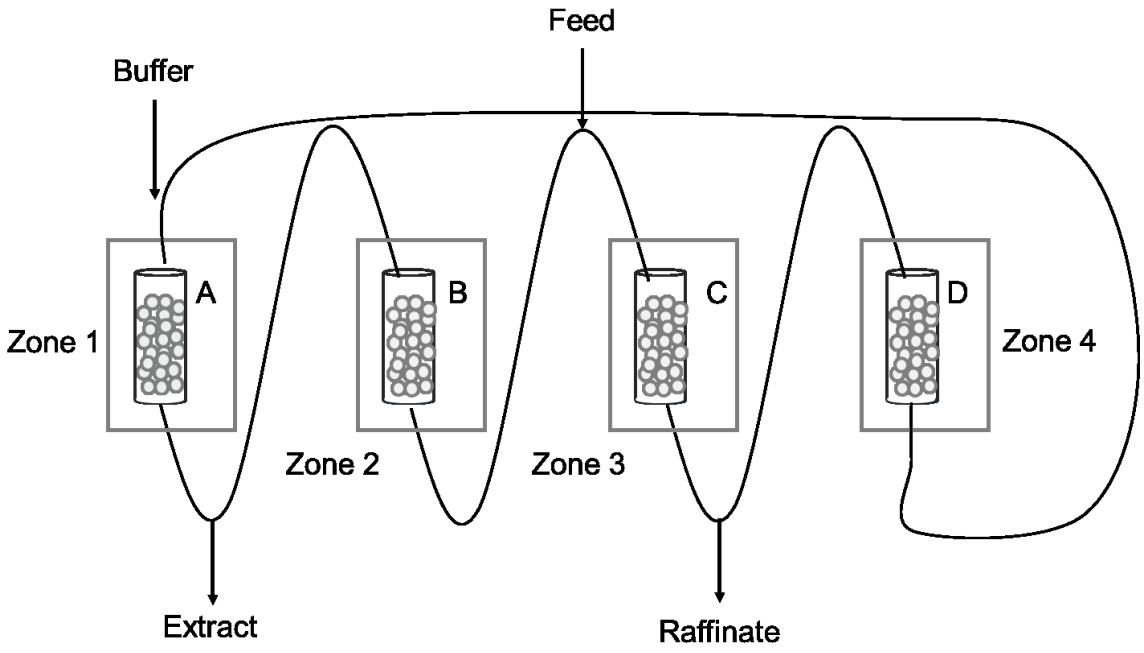

Figure 6. Simulated Moving Bed Separation Zones
Simulated moving bed chromatography splits the long continuous bed in true moving bed chromatography into smaller segments connected in sequence. Inlet and outlet ports separate the columns into zones, each having a specific role in the separation.[4]

In zone 1, the buffer pushes the slower-moving component off of the columns into the extract, simultaneously equilibrating the columns. Zone 2 includes columns between the extract and the feed. Its role is to retain the slower-moving

component while the buffer pushes the faster-moving component forward. The feed enters into zone 3, which includes columns until the raffinate port. The beginning of zone 3 typically remains saturated with feed, while the rest allows the faster-moving component to separate and exit in the raffinate. Zone 4 is optional and can be used to regenerate buffer and collect any material continuing past the raffinate, further purifying it. The inlets and outlets move forward with every switch, thereby switching which zone each column is in.[4]

STARTUP & STEADY STATE CONFIGURATIONS

To help the reader visualize how separation occurs in SMB chromatography, the steps beginning from startup will be described in detail in the following sections. In the figures below, each zone will contain one column at a time. Dark purple will represent the feed unseparated in the system, red the slower-moving component, blue the faster-moving component, and yellow the buffer.

It is important to note that the description below applies most accurately to separations whose elutions do not require a desorbant other than the original buffer. It is a helpful starting point in order to understand other continuous system setups, and can be modified for application to separations that do require desorbants, as will be discussed subsequently.

Before 1st Switch At startup, the system is equilibrated in buffer. The buffer continues saturating zone 1, column A, and part of this buffer exits in the extract while the rest continues through zone 2, column B. In the meantime, the feed saturates the beginning of zone 3 (the top of column C), while the faster-moving component starts to gain on the feed front at the bottom of the column. A portion of the faster moving component exits in the raffinate, and the rest is collected by zone 4, column D. The system approaches the state in Figure 7, which is achieved just before the first switch occurs.[4]

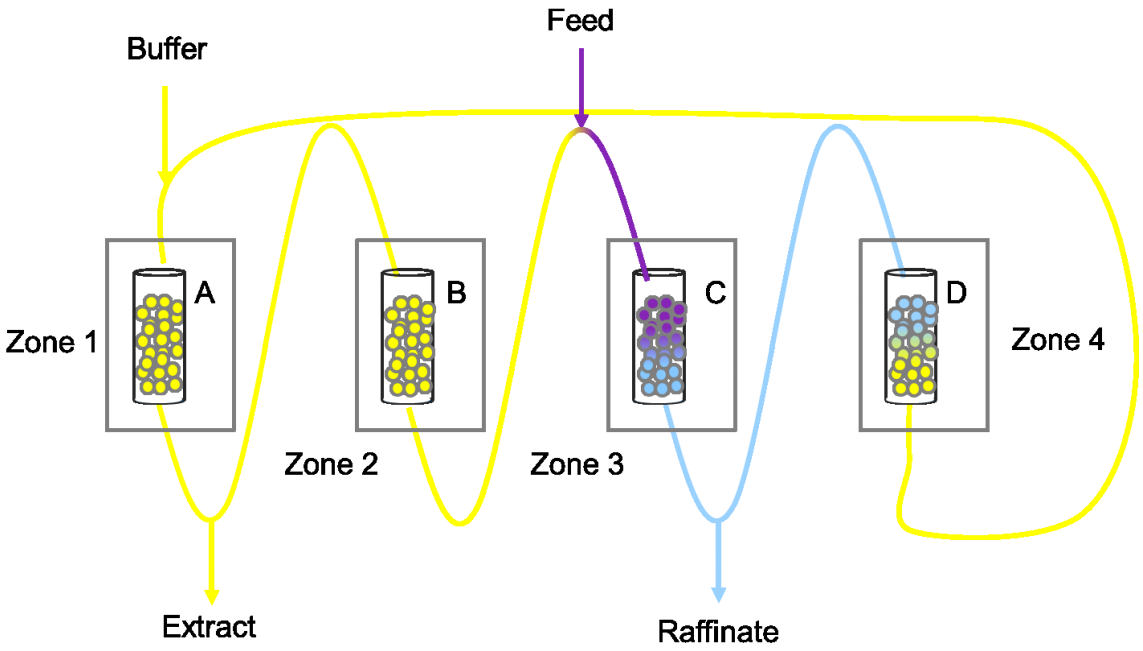

Figure 7. Simulated Moving Bed System Just Before 1st Switch
At startup, the buffer (yellow) equilibrates column A (zone 1). Some continues into the extract and some onto column B (zone 2). Feed (purple) saturates the top of column C (zone 3) and separation begins at the bottom. Part of the faster-moving component (blue) exits in the raffinate and the rest moves onto column D (zone 4).[4]

Between 1st and 2nd Switches Immediately after the first switch, the inlets and outlets have simply changed positions in the direction of fluid flow, as shown in Figure 8.[4]

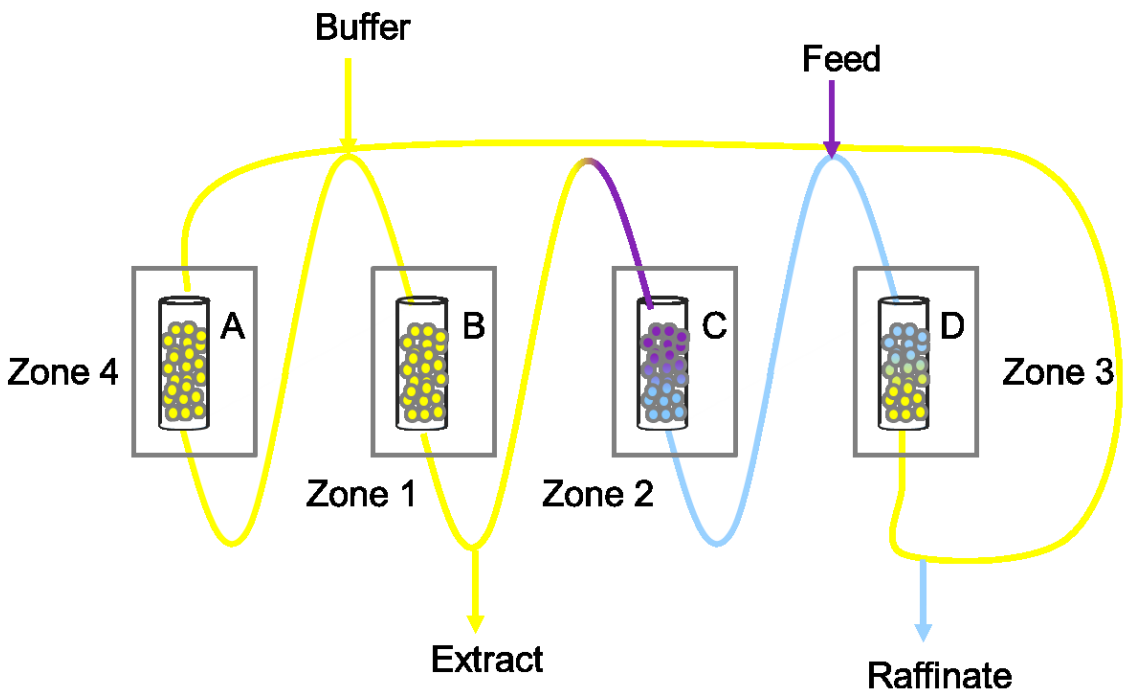

Figure 8. Simulated Moving Bed System Just After 1st Switch
Just after the first switch, column C, still containing feed (purple) from its time in zone 3, enters into zone 2, where the buffer (yellow) pushes the faster-moving component (blue) onto column D (zone 3), leaving the slower-moving component (red) behind. In the mean time, the faster-moving component left on column D from the previous configuration is pushed into the raffinate, while the feed saturates the top of the column.[4]

At this point, no material has built up in column B, and so the extract will still only consist of buffer. The buffer that makes it onto zone 2 pushes the feed on column C towards zone 3, leaving the slower-moving component behind. The feed simultaneously saturates the top of column D and pushes the faster-moving component into the raffinate and onto column A.[4]

If the switch time is chosen long enough so that all of the faster-moving component has left column C, but short enough so that the slower-moving component does not reach column D, the system will approach the state in Figure 9, which is achieved just before the second switch.[4]

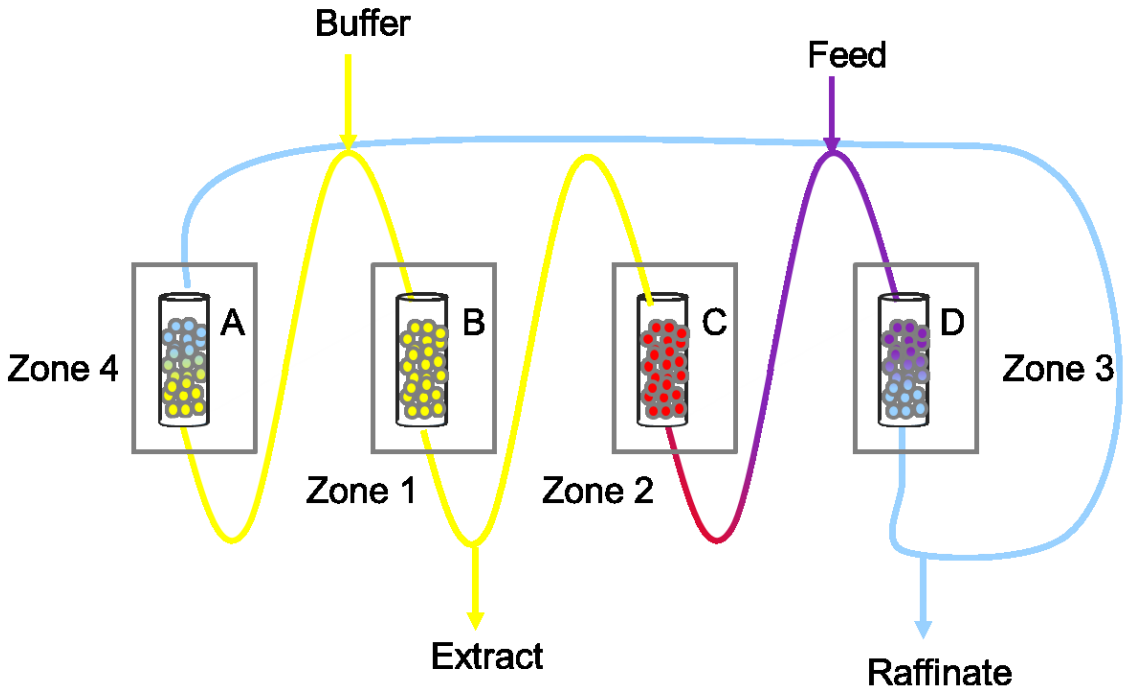

Figure 9. SMB System Just Before 2nd Switch
Just before the second switch, all of the faster-moving component (blue) on column C (zone 2) has made it onto column D (zone 3), but the slower-moving component (red) is still retained. The feed (purple) saturates the top of column D while the faster-moving component (blue) exits in the raffinate or is collected onto column A (zone 4), as before.[4]

Again, immediately after the second switch occurs, the system can be represented simply with the inlets and outlets having changed positions, as in Figure 10.[4]

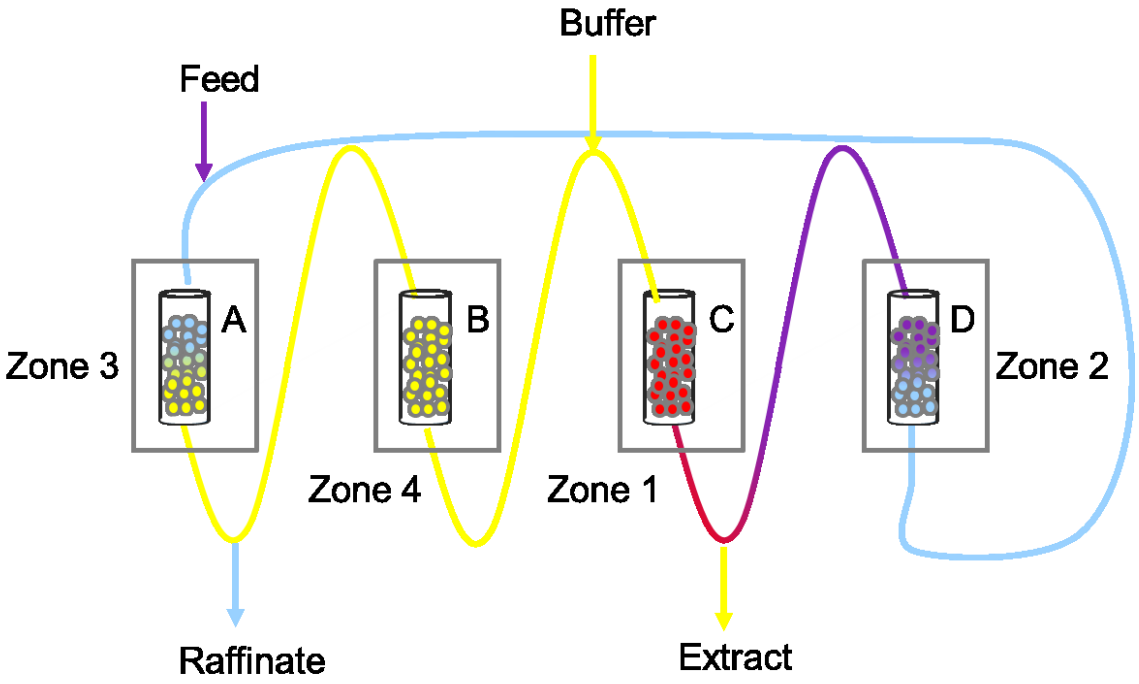

Figure 10. SMB System Just After 2nd Switch
Just after the second switch, the slower-moving component (red) retained on column C makes it into zone 1, where the buffer (yellow) begins pushing it into the extract. Simultaneously, the faster-moving component (blue) on column D (zone 2) is pushed onto column A (zone 3), and that on column A is pushed into the raffinate.[4]

Between 2nd and 3rd Switches Now that the slower-moving component has reached zone 1 on column C, it finally starts to accumulate in the extract. As previously, the buffer that makes it into zone 2 pushes the feed left on column

D forward, leaving the slower-moving component behind. Simultaneously, the feed saturates the top of column A and pushes the faster-moving component into the raffinate and onto column B in zone 4. The system is now in steady state and the same pattern should continue.

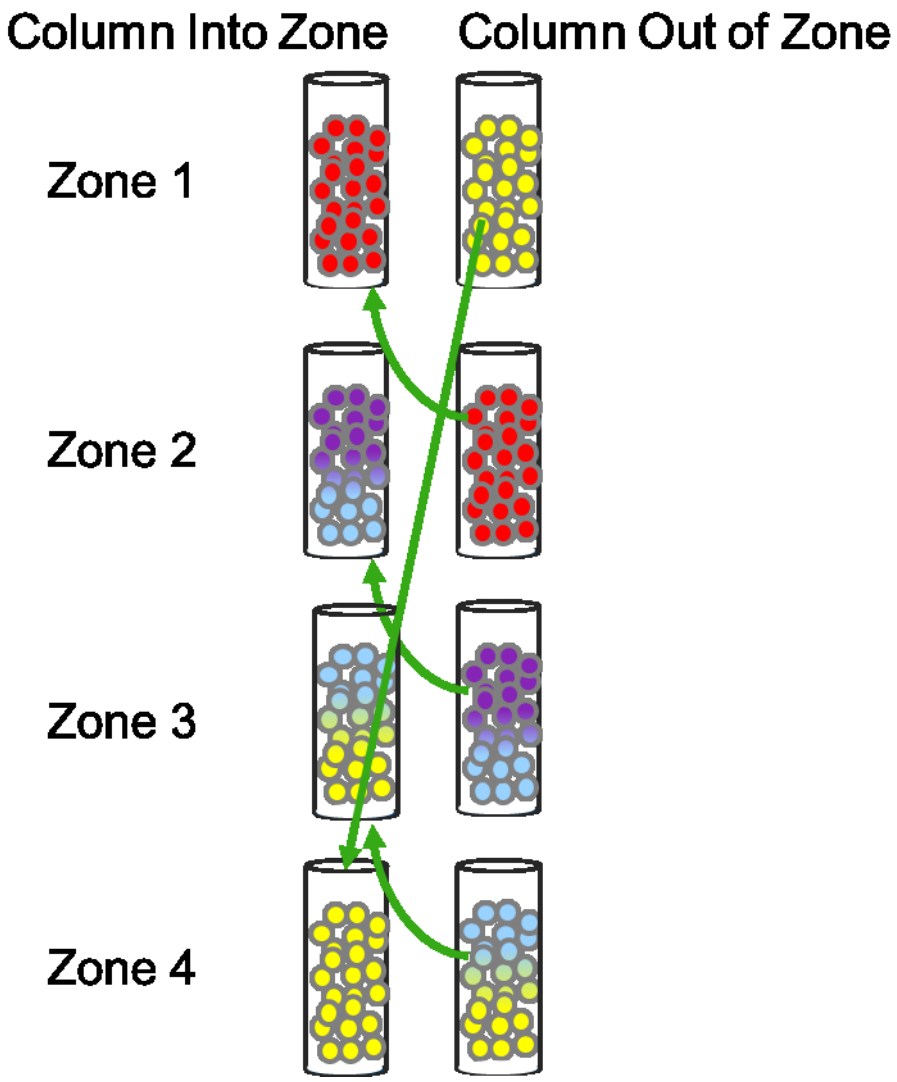

Figure 11. Steady State SMB System Configurations
After the simulated moving bed system reaches steady state, regardless of which columns are in each zone, the system will fluctuate between the conditions shown, with red representing the slower-moving component, purple the feed, blue the faster-moving component, and yellow the buffer.

Switch Time Selection It is now clear why the switch time must be carefully chosen. If it were too short, some faster-moving component would remain on the bed in zone 2 and be switched into zone 1, where the buffer would push it into the extract. If the desired product were meant to be in the extract, this would decrease purity; if it were meant to be in the raffinate, a short switch time would diminish the yield. Conversely, if the switch time were too long, the slower-moving component in zone 2 would reach zone 3, and both components would move in the same direction and exit in the raffinate, rendering the separation ineffective.

SMB IN PRACTICE

Cadence BioSMB System The Cadence BioSMB PD System from Pall, was used for all continuous studies discussed in the following sections. This system has seven pumps, eight inlet ports, six outlet ports, and a valve block that can accommodate up to sixteen columns.

A screen shot of the system's software is shown in Figure 12. The method that each column must undergo should be dictated in the top section: Each step must have a specified name, inlet, outlet, and two of either the fluid volume, flow rate, or time. The software will then calculate the division of these steps over time

based on the number of columns used. As the process runs, the columns (designated by C1, C2, and C3 in the chronogram below the table) rotate clockwise, making it clear which step each column is experiencing. Tubing can connect the outlet ports to collection vessels, and the system can run as many cycles as desired consecutively.

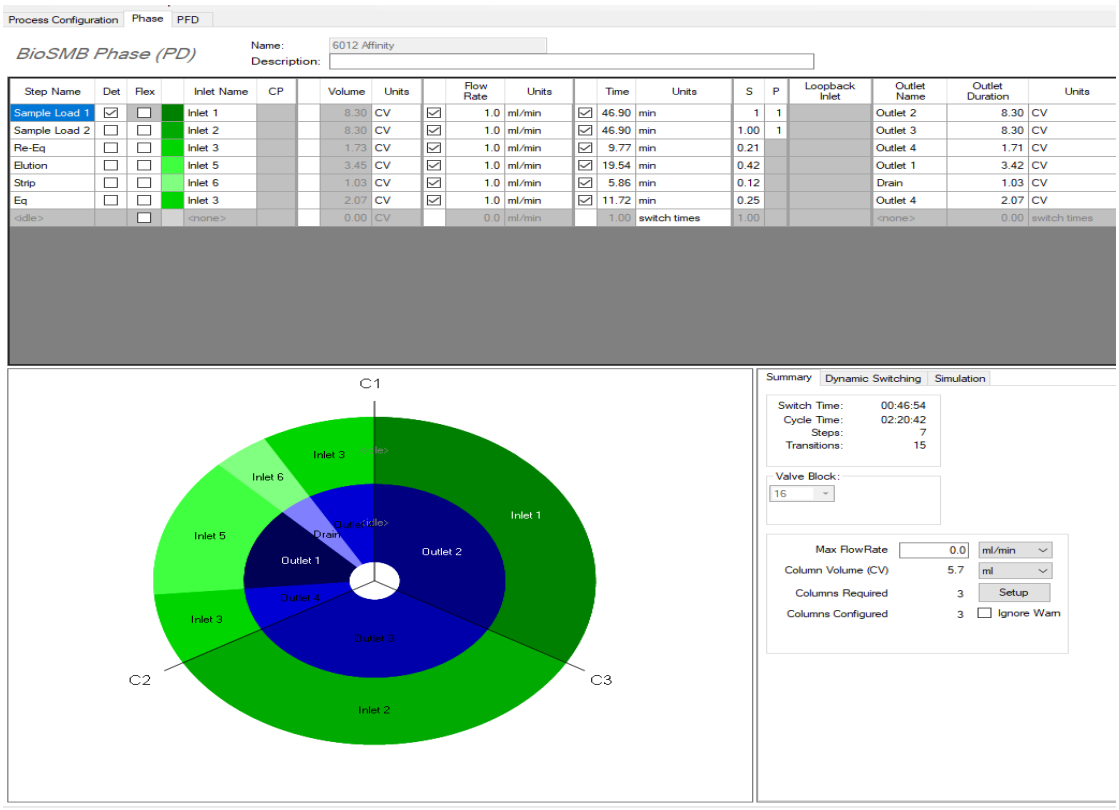

Figure 12. Cadence BioSMB System Software Example Screenshot
In the method design page for the simulated moving bed system's software, the method is defined in the top rectangle. As the process runs, the columns move clockwise in the chronogram below, signifying which steps they are experiencing.

ENZYME POLISHING WITH CONTINUOUS HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

The first process developed on the premise of SMB chromatography was a polishing step for a relatively hydrophobic enzyme, targeting the removal of its more polar inactive form. This enzyme has a molecular weight of 50 kDa and a pI of 4.7.

HOW HYDROPHOBIC INTERACTION CHROMATOGRAPHY WORKS

Hydrophobic interaction chromatography (HIC) resins reversibly bind proteins with hydrophobic surfaces. The degree to which these proteins are retained depends on the salt concentration of the buffer used: High concentrations encourage the proteins' interaction with the resin by decreasing their tendency to remain solvated in the buffer, while lowering the salt concentration has the opposite effect. For this reason, HIC separations often employ gradient elutions, starting with high salt to preferentially bind the hydrophobic proteins, and decreasing the concentration only after the hydrophilic components have exited the column, allowing the hydrophobic proteins to elute separately.[5]

BATCH PROCESS DEVELOPMENT

A batch method using gradient elution was previously designed for the separation of these enzymes. Because the active species is more hydrophobic than the inactive, it was collected at the end of the elution at the low salt concentrations. Unfortunately, gradient elutions are very complicated to incorporate into SMB setups, and so it was necessary to design a new batch method with successful isocratic elution before a continuous process could be designed.

Quantifying the Success of a Separation A previously designed HPLC HIC method was used to analyze the purity and yield of the fractions from batch testing. A standard chromatogram including peaks for both enzyme forms was used as reference. Purities were calculated by dividing the area of the active enzyme's peak by the total area of the two peaks; yields were calculated simply via mass balances.

Resin Selection First, three HIC resins were tested to determine which would best separate the two species: Three 0.66 cm diameter Omnifit columns were packed with butyl sepharose, polypropylene glycol, and MacroPrep Methyl HIC resin (Methyl), respectively. These resins were tested using the previously

designed batch separation with a gradient elution. The results suggested that Methyl was the most effective resin for this separation, as it yielded the only chromatogram with a clear shoulder for the later-eluting active species, as shown in Figure 13. Thus, methyl was used for all subsequent testing.

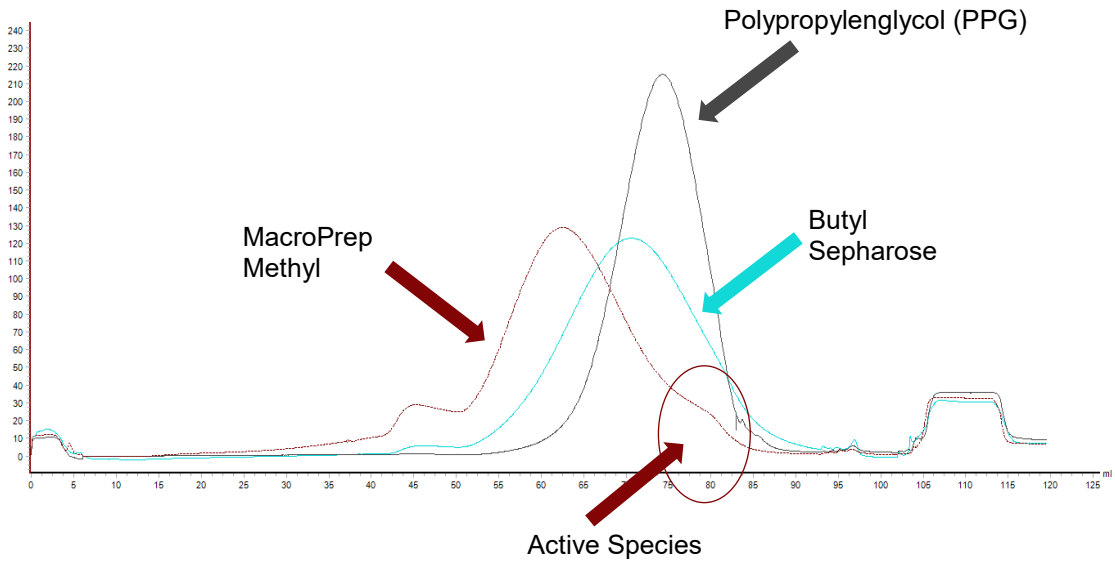

Figure 13. Gradient Elution Batch Chromatograms Comparing HIC Resins The UV 280 readings for each resin in the previously designed batch method were overlaid. Methyl was the only one to produce a distinct shoulder for the later-eluting active species, and therefore was selected as the resin for all subsequent testing.

Selecting Flow Rate and Sample Volume After the resin was selected, multiple combinations of flow rates, sample volumes, load protein concentrations, and buffer salt concentrations were tested. It was found that a sample load/elution flow rate of 0.684 mL/min (5 minute residence time) and a

2 mL sample volume provided adequate separation among the varying salt concentrations, and were therefore used for all subsequent testing.

Determining Isocratic Buffer Salt Concentration The relationship between a protein's concentration in solution and the fraction of protein bound to the resin can be described by the protein's isotherm for a given salt concentration. Isotherms typically begin with a linear portion, where concentration in solution is proportional to fraction bound. However, after a certain point, the resin's capacity is reached and no more protein can bind, so the isotherm plateaus. It is desired for a separation to occur in the initial linear region so that the resin is not overloaded, and it can effectively retain the proteins.

In order to select a salt concentration for this particular separation, isotherms were constructed for sodium sulfate ranging from 0 to 600 mM. Protein solutions ranging from 0.25 to 5.0 mg/mL were incubated with a constant volume of Methyl at the varying salt concentrations and left rotating overnight to ensure they reached equilibrium. The resulting concentrations of enzyme in solution were measured, and these samples were analyzed on HIC HPLC to determine how much of each variant had bound to the resin. The mass of each enzyme

bound per volume resin was plotted against the final protein concentration in solution, producing the isotherms for each species.

The only isotherms with a linear region at practical protein feed concentrations (not below most equipments' limit of detection) were those for 200 and 300 mM sodium sulfate, shown in Figure 14. Those for higher salt concentrations were too steep and required unmanageably low protein load concentrations.

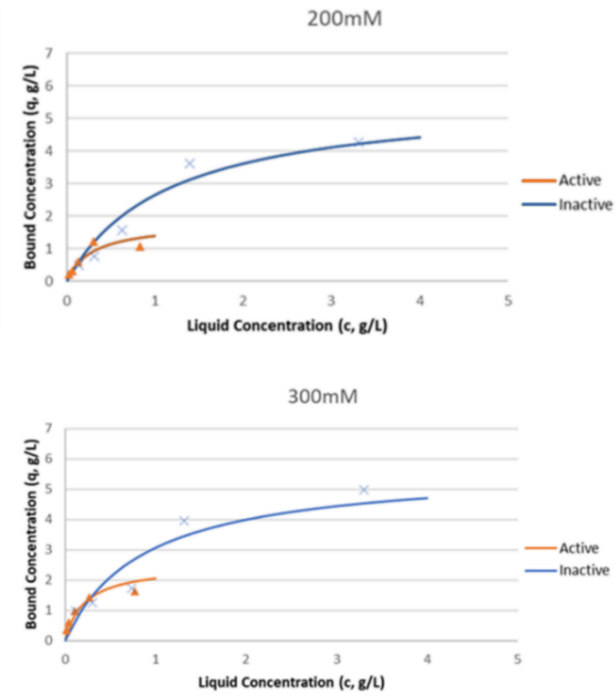

Figure 14. 200 & 300 mM Sulfate Methyl HIC Isotherms for Active and Inactive Enzyme Species
Isotherms for 0-600 mM sodium sulfate were constructed to understand how buffer salt concentration influenced protein binding as a function of protein concentration in solution. The isotherms for 200 and 300 mM sodium sulfate yielded a linear region at practical feed concentrations. (Figure created by Chris Thompson)

From this information, a final set of batch runs was conducted at salt concentrations ranging from 150 to 350 mM sodium sulfate. Analysis of the fractions from these tests on HIC HPLC showed that 250 mM sodium sulfate provided the best separation of the two species, and so this salt concentration was selected for all subsequent testing.

Selecting Feed Protein Concentration Finally, after the salt concentration was selected, it was established that increasing the load concentration up to 2 mg/mL did not significantly affect the HIC HPLC fraction data, and so this feed concentration was selected.

Addition of Elution Step HPLC data from the isocratic elution fractions revealed a similar pattern to that from the original gradient elution, with the inactive species exiting before the active species in the flowthrough, illustrated in Figure 15. However, without the use of a salt-free solution at the end of the wash, a significant portion of the active species remained bound to the resin. For this reason, the salt gradient had to be replaced with an additional elution step using a salt-free desorbant.

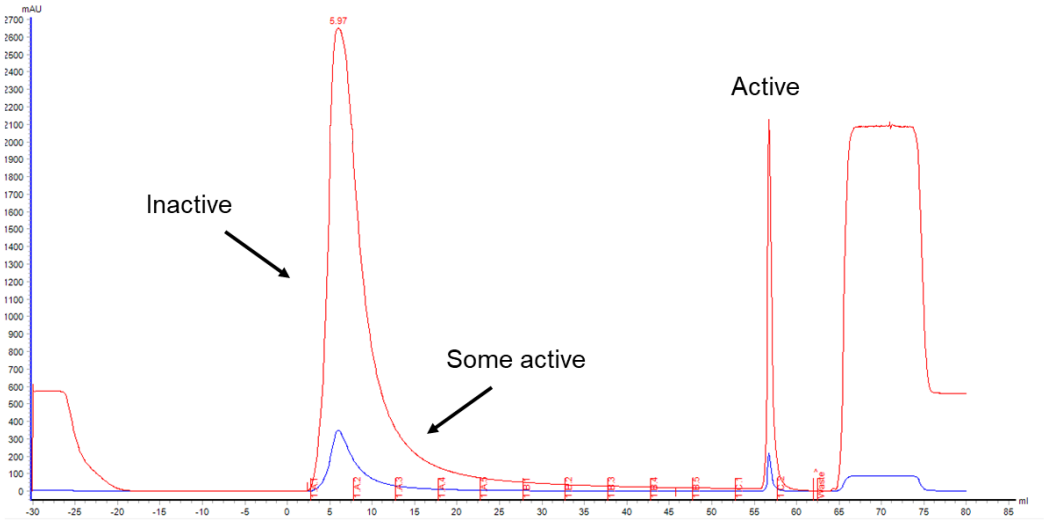

Figure 15. HIC Elution Sequence of Active and Inactive Species
The elution pattern from the isocratic elution was similar to that of the previously used gradient elution, with the inactive enzyme eluting first. However, only a small portion of the active species eluted in the tail of the first peak when the same buffer was used. A separate salt-free desorbant was necessary to elute the rest.

Batch Method The final method selected (which produced the chromatogram in Figure 15) is shown in Figure 16, where CV (column volume) represents fluid volume normalized to the volume of resin in the column.

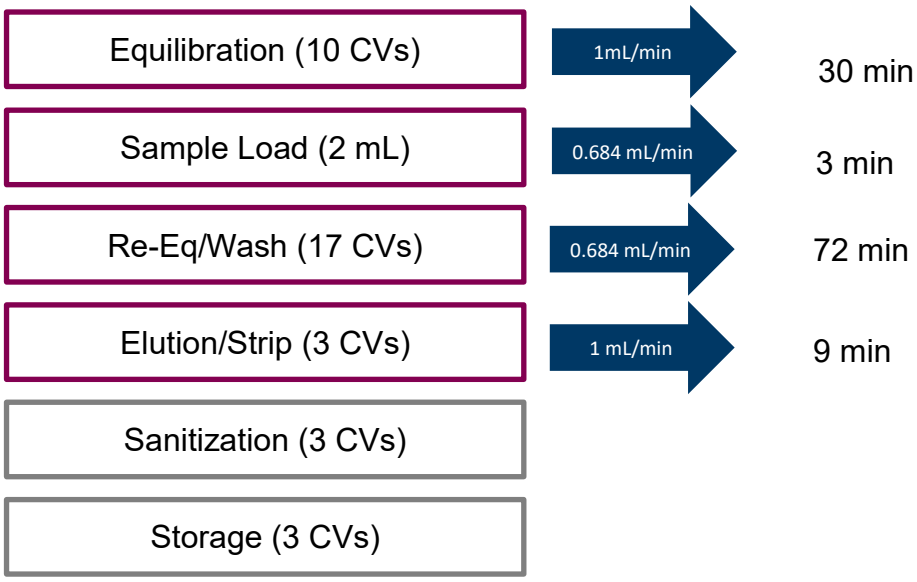

Figure 16. Batch Method Selected for HIC Separation
The parameters selected for this separation were a flow rate of 0.684 mL/min, a 2 mL sample volume at 2 mg/mL, and 250 mM sodium sulfate. The durations of each step were dictated by the buffer volumes required for UV and/or conductivity readings to stabilize in the chromatogram.

Batch Results It was found that the purity in the tail of the first peak and the purity in the desorbant peak were correlated for the different batch runs; for simplicity, comparisons were made on the basis of the elution pool's purity. The most successful separation reached a 97% purity and resulted from using 250 mM sodium sulfate at a 5-minute residence time (0.684 mL/min) with a 2 mL sample volume at 2 mg/mL.

CONTINUOUS PROCESS DEVELOPMENT

As a reminder, the standard setup of an SMB chromatography system is depicted in Figure 17.

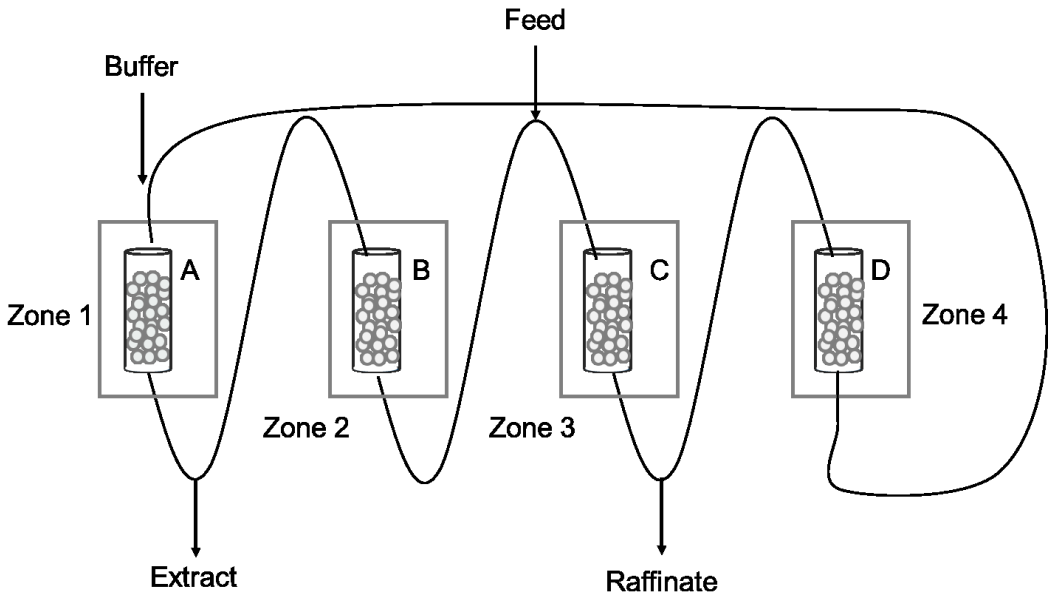

Figure 17. Standard SMB Chromatography Setup[4]

Three modifications were made to this setup to accommodate the specific requirements of our separation.

Increasing the Length of Zone 2 Because the elution profiles from the HPLC HIC data for the two enzymes overlapped, it was decided to improve purity by increasing the total bed height between the feed and the extract. This was done by adding two more columns in series in zone 2.

Disconnecting the Elution Step In order to not disrupt the flowthrough separation by mixing desorbant into zones 2-4, zone 1 was removed from series and the elution collected as extract independently.

Adding a Drain The final modification to the standard setup was the addition of an outlet after the raffinate. This drain was added to avoid recycling the buffer, thereby simplifying the process's approval by regulatory organizations if the process were to be scaled up.

Incorporating these three modifications, the setup for this separation is depicted in Figure 18.

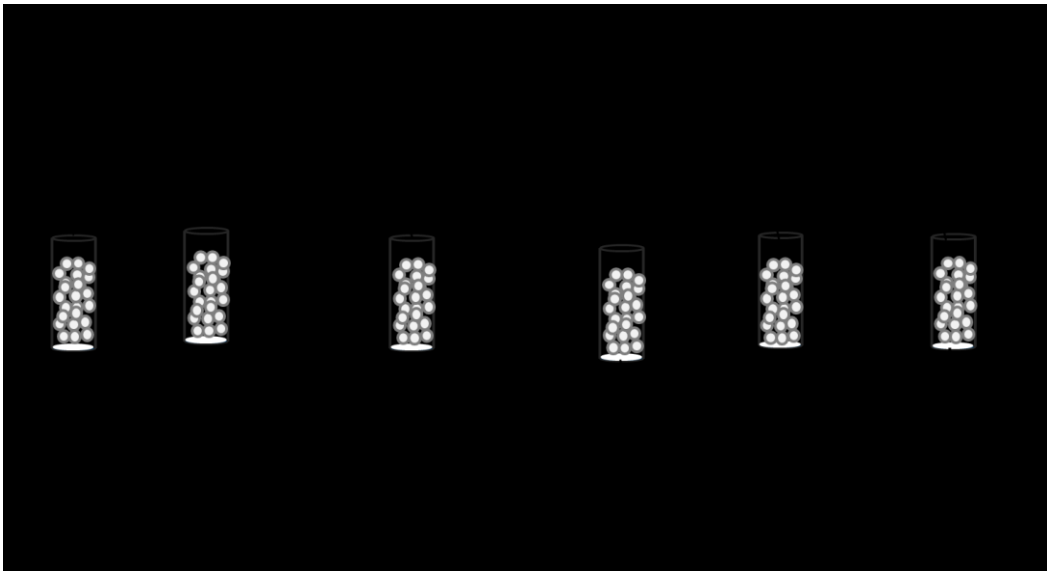

Figure 18. HIC Separation Adapted SMB Setup
The final SMB setup for this separation differed from the standard description in three ways: It included two additional columns in zone 2 to increase product purity, the additional elution step was removed from series to not disturb the flowthrough elution, and a drain was added to prevent recycling buffer.

After having defined the system setup, the flow rates, switch time, and feed concentration could be chosen.

Selecting Flow Rates Batch studies showed that a flow rate of 2 mL/min yielded adequate separation. However, for the size of the columns being used, this flow rate was relatively high; for the long periods of time that the continuous system would run, it was more cautious to slightly lower the flow rate. The buffer rate was therefore selected to be 1 mL/min and the feed 0.2 mL/min, resulting in a total 1.2 mL/min into zone 3. In order to maintain a constant volume in the

continuous system, the raffinate and drain flow rates were each designated as 0.6 mL/min. The desorbant and extract flow rates were both 1 mL/min.

Before the switch time could be selected, differences between the batch and continuous setups needed to be accounted for. Differences in both the rate and distance travelled would affect the time at which the proteins elute.

Parameters Affecting Rate of Protein Movement The proteins' velocities result from the forces acting on them: gravity, the forward force of the buffer, and the retention from the resin.

- Gravity obviously does not vary between the batch and continuous setups.
- The force from the buffer can be maintained between setups by using similar flow rates. Otherwise, changing the flow rate would produce equivalent changes in the proteins' rate of travel.
- The resin's activity can be described by the previously constructed isotherms. If batch studies are conducted in the linear region of the isotherm, selecting a load concentration for the continuous system also in this region should yield comparable retention from the resin and not affect the protein's rate of travel.

Parameters Affecting Distance Travelled Differences in the distance travelled between batch and continuous setups must also be accounted for when trying to predict at what time the two proteins will elute. In addition to differences in the systems' internal path lengths, the mechanism by which loading occurs in the two setups should also be noted: In batch, the bulk of the feed reaches the top of the column at the start of the sample load, and from that point only needs to travel through the bed of resin for the sample load to be complete. On the other hand, in the continuous setup, the same mass of protein enters at a much lower concentration with each molecule starting from the top of the column throughout the switch time. Therefore, the total time required for the sample load is expected to be slightly longer than that in batch.

Selecting Preliminary Switch Time The HPLC HIC data from the fractions of the most successful batch run was used to construct elution profiles for each species (mass of specific enzyme eluting against elution volume). These graphs showed the peak maximum for the inactive species occurring at approximately 7 minutes. Therefore, it seemed likely that a 7-minute switch time would allow the majority of impure product to flow through without losing a significant amount of active species. However, because the flow rate selected was slightly lower than that of the batch setup, and because of the previously

mentioned difference in loading between the two setups, it was expected that the switch time would need to be slightly longer.

Selecting Feed Concentration For simplicity, it was decided to maintain the same ratio of mass enzyme/volume resin used in batch studies, and therefore a feed concentration of 3 mg enzyme/mL solution was selected.

Adjusting Switch Time After all of the necessary variables were defined, this setup was run and the elution pools analyzed using the same HPLC HIC analysis used on fractions from the batch studies. As predicted, separation with a 7-minute switch time was not as successful as desired. While it was expected that a slightly longer switch time would yield optimal results, 5-minute, 9-minute, and 11-minute switch times were all tested. For each switch time, the system was allowed to reach steady state, and the subsequent elution pools were analyzed for yield and purity.

Continuous Results Figure 19 shows the yields and purities of the elution pools from the different switch times. It is clear that the 9-minute switch time provided the best tradeoff between the two (41% yield and 84% purity).

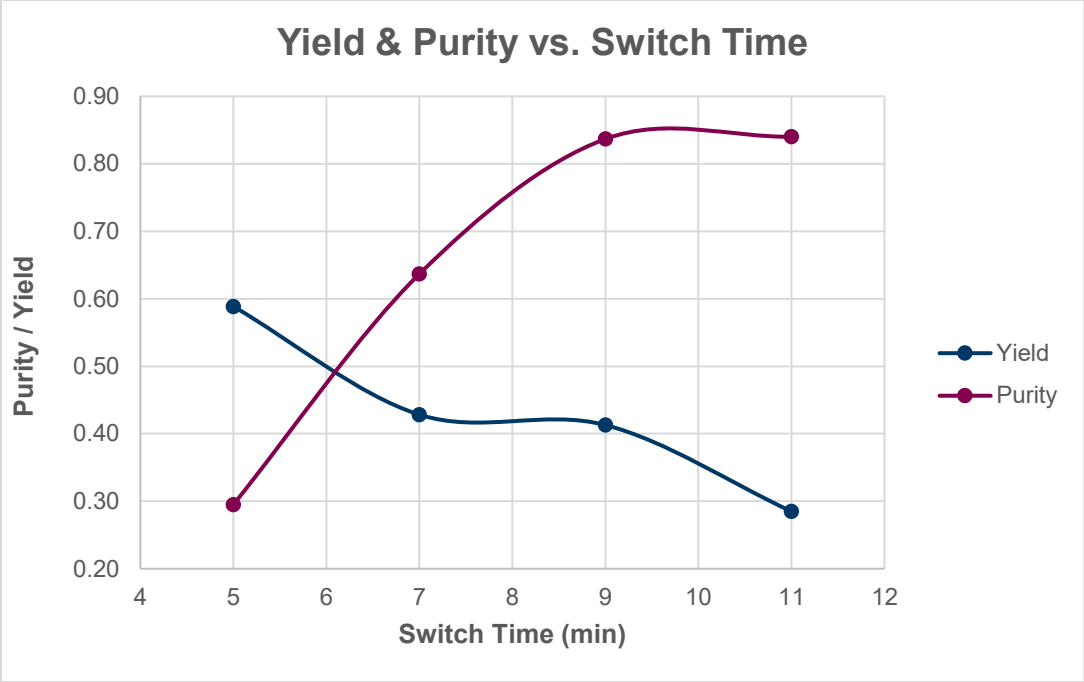

Figure 19. HIC Switch Time Purity and Yield Comparison
The elution pools' yield and purity were plotted against switch time. The 9-minute switch time produced the best tradeoff between purity and yield.

DESIGN OF A CONTINUOUS ENZYME CAPTURE STEP

The second process developed on the premise of SMB chromatography was the capture step for the same enzyme.

BATCH PROCESS DEVELOPMENT

A 0.66 cm diameter Omnifit column was packed with 1.95 mL of customized affinity resin.

Determining Resin Binding Capacity In order to determine how much material could be purified given a volume of resin, a dynamic binding capacity study was conducted, in which an overload (20 mg enzyme/mL resin) was fed onto the column. The flowthrough was collected and fractions analyzed to determine the concentrations of enzyme that exited before the elution step. These concentrations were then plotted against the total mass of protein loaded at that time, and the results, shown in Figure 20, yielded a sigmoidal curve with an inflection point at the resin's binding capacity, approximately 9 mg enzyme/mL resin.

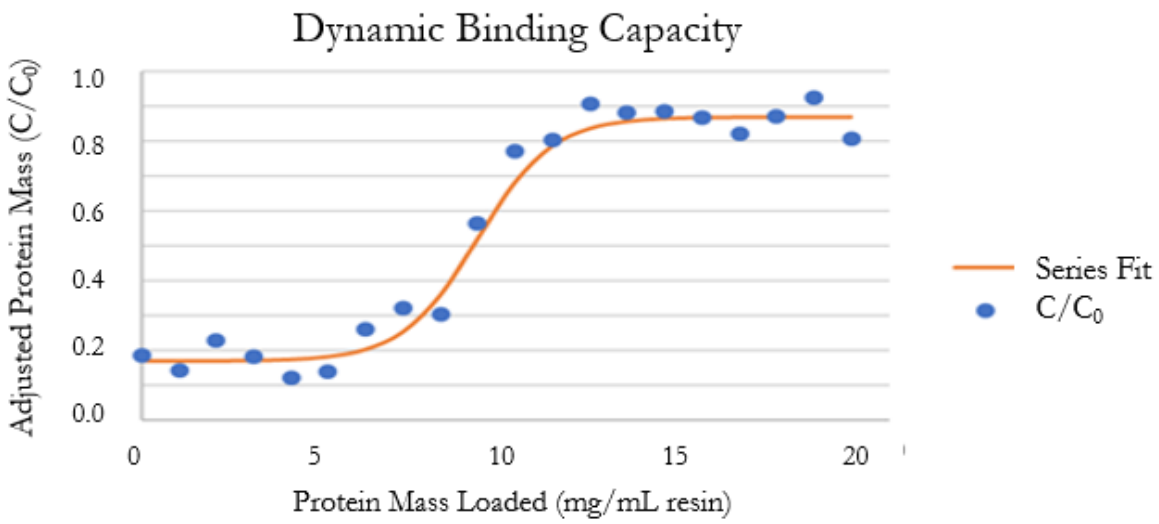

Figure 20. Affinity Dynamic Binding Capacity Curve A dynamic binding capacity study was conducted in which a protein overload was pumped over the resin. Enzyme presence in flowthrough was recorded to determine at what mass loaded the protein was no longer binding. The binding capacity of the resin was found to be 9 mg enzyme/mL resin. To be safe, subsequent tests loaded to 5 mg enzyme/mL resin.

It was decided to load the columns in subsequent testing to 5 mg enzyme/mL resin to ensure no loss of the enzyme.

Selecting Elution Buffer Five different desorbants, listed in Figure 21, were tested for the elution step. The desorbant yielding the highest recovery (30% propylene glycol/ 1M Arginine HCl) was selected for elution.

Desorbants Tested

- 1 M Arginine HCl
- 20% Propylene Glycol/1M Arginine
- 30% Propylene Glycol/1M Arginine
- 20% Hexylene Glycol
- 40% Propylene Glycol/1M Arginine

Figure 21. Desorbants Tested for Affinity Elution
Five desorbants were tested and their enzyme recoveries compared. 30% propylene glycol/1M arginine provided the highest yield and was selected for subsequent testing.

Batch Method The batch method selected is shown in Figure 22.

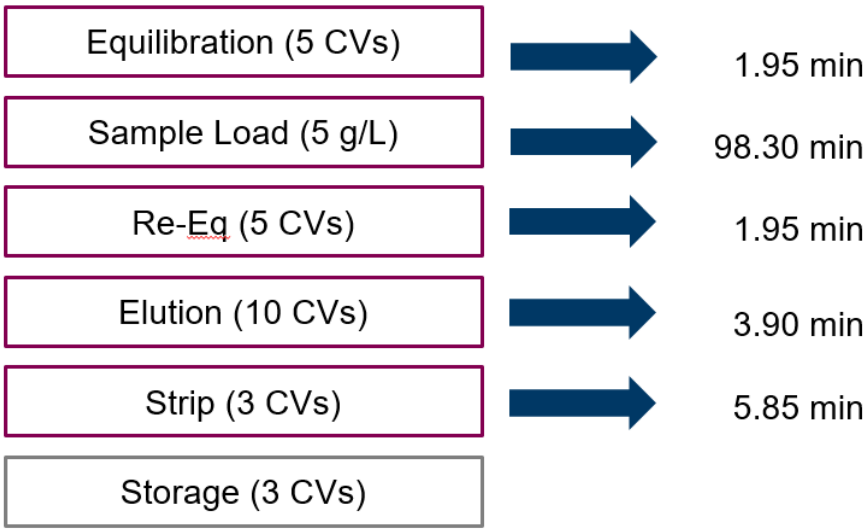

Figure 22. Batch Affinity Method Selected
The most successful batch method used flow rates of all approximately 1 mL/min and loaded to 5 mg of enzyme/mL resin.

As previously described, the length of the equilibration, re-equilibration, elution, and strip steps were determined from when UV and conductivity readings stabilized in batch testing. The length of the sample load was dictated by loading to 5 mg enzyme/mL resin. Flow rates were selected to be as fast as reasonable for each step, all approximately 1 mL/min. Regardless, the protein concentration in the feed was so low that the sample load took approximately twice as long as the rest of the steps combined.

Batch Results The results from the most successful batch runs yielded a 71% purity and a 72% yield. These numbers were determined based on reverse phase HPLC analysis of the elution pool.

CONTINUOUS PROCESS DEVELOPMENT

Modifications to Standard SMB Design The defining difference between this affinity separation and the flowthrough separation described in standard SMB chromatography was that a separate desorbant was required to elute the protein of interest. The absence of a flowthrough separation meant that, because the resin's binding capacity was not exceeded, the flowthrough from the sample load did not contain any enzyme and could be directed straight to waste. In addition, the purity of the product in the elution step did not depend on the bed

height. It was therefore no longer important to connect the columns in series; each column could undergo one step without disrupting the others.

Continuous Process Because of the aforementioned differences from standard SMB chromatography, designing a continuous process for this affinity separation boiled down to dividing the total time required for the batch method by the number of columns available. Because the sample load took twice as long as the other steps combined, it was clear that two of the three columns should be loaded while the third experiences all of the other steps. This process is depicted in Figure 23.

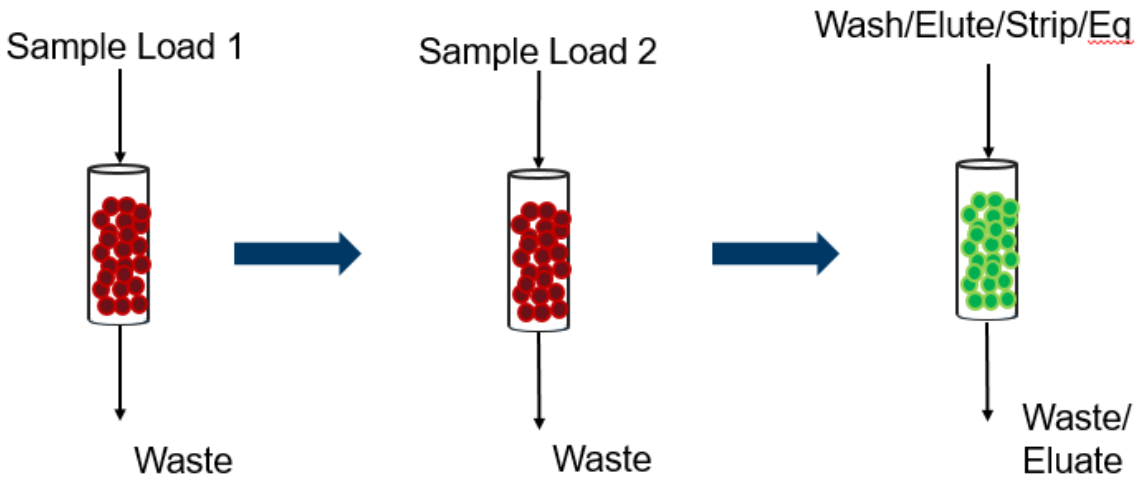

Figure 23. Continuous Affinity SMB Process
The resin's binding capacity was not exceeded, and purity did not depend on distance travelled down the resin, so flowthrough from the sample load could be directed to waste and the columns did not need to be connected in series. The sample load took twice as long as the other steps combined, and so two of the three columns underwent sample load while the third experienced the rest of the method.

This method was saved into the Cadence BioSMB software, shown in the screenshot in Figure 24. The two sample load steps were first undergone by columns 3 and 1, while column 2 started with the re-equilibration/wash, elution, strip, and equilibration steps.

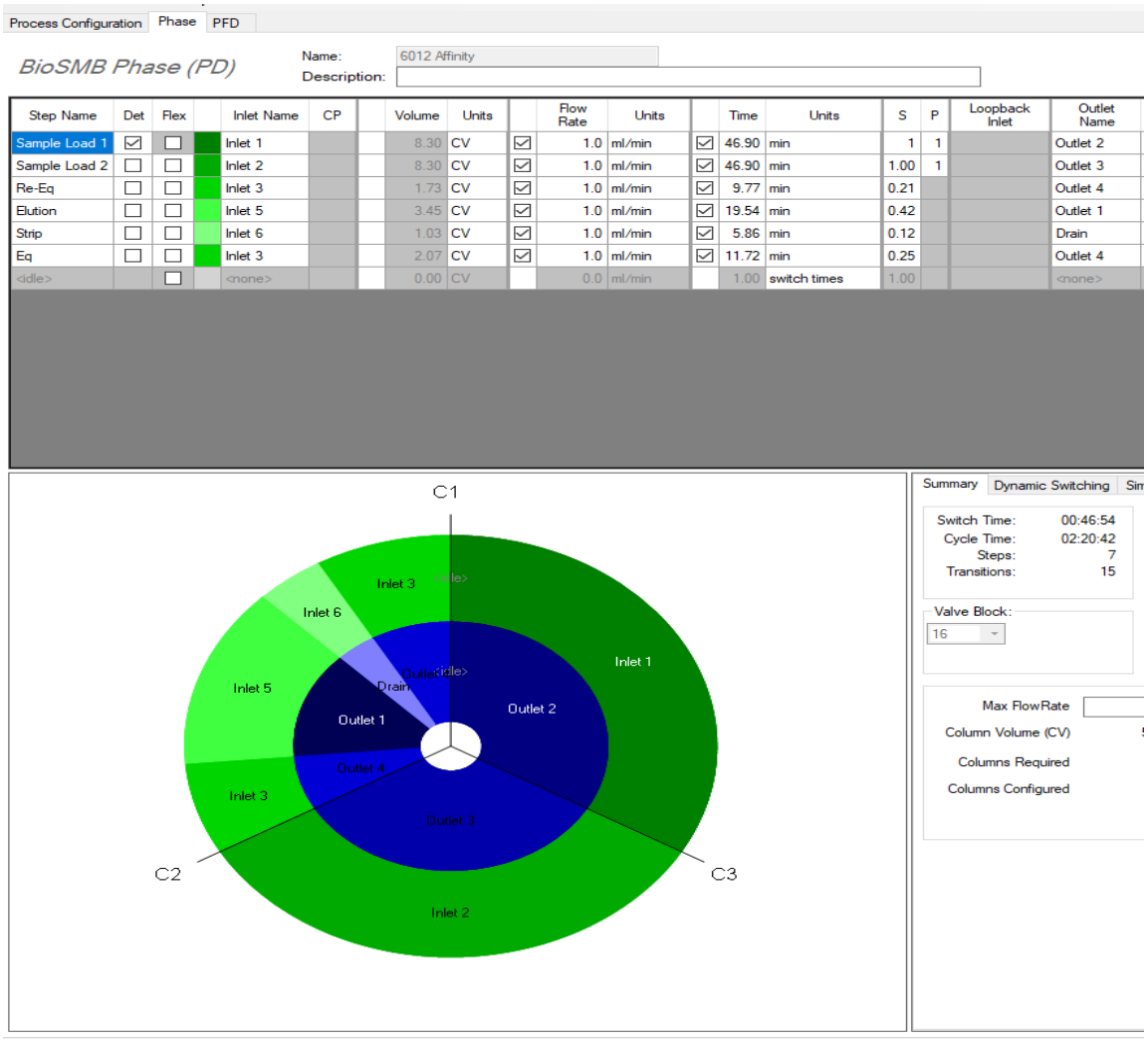

Figure 24. Affinity BioSMB Software Screenshot

Continuous Results The purity from the continuous run elution pool reached 87% with a 69% recovery. These numbers were determined using the same reverse phase HPLC analysis utilized for the batch fractions' analysis.

CONCLUSIONS

Continuous processes for both a polishing separation using HIC and an affinity separation using a customized resin were successfully designed and produced high purity and acceptable yield. These results, accompanied by more efficient resin usage, encourage the application of SMB chromatography to many other separations. With a large fraction of early stage manufacturing costs being attributed to resin supply, the benefits of continuous chromatography could prove significant in the biopharmaceutical industry.

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CURRICULUM VITAE

Michelle Bahri was born in 1995 in the United States.

She attended Johns Hopkins University for both her bachelor's and master's studies in chemical and biomolecular engineering. Throughout her time as an undergraduate, Michelle worked in Dr. Michael Betenbaugh's research lab, where she took part in three projects and was selected to co-author a short section in Perry's Handbook about simulated moving bed chromatography. She obtained her bachelor's degree with honors in May 2017.

During her master's studies, Michelle joined Dr. Jamie Spangler's lab as one of her first students and studied the development of bispecific antibodies to reduce metastasis for certain types of cancers. Michelle also served as the secretary for the Johns Hopkins chapter of the society of women engineers (SWE), learned how to create music of her own in Ableton Live through workshops at the digital media center, and has significantly improved her Italian-speaking abilities.

After her year of master's courses, she took part in a co-op of six months at AstraZeneca/MedImmune, where she conducted the work described in this essay. Upon approval, she will graduate with her master's degree in May 2019.